

## Molecular characterization of a partitivirus from *Ophiostoma himal-ulmi*

Lauren J. Crawford · Toba A. M. Osman ·  
Frank P. Booy · Robert H. A. Coutts ·  
Clive M. Brasier · Kenneth W. Buck

Received: 28 September 2005 / Accepted: 17 October 2005  
© Springer Science+Business Media, LLC 2006

**Abstract** The complete nucleotide sequences of two double-stranded (ds) RNA molecules, S1 (1,744 bp) and S2 (1,567 bp), isolated from an isolate HP62 of the Himalayan Dutch elm disease fungus, *Ophiostoma himal-ulmi*, were determined. RNA S1 had the potential to encode a protein, P1, of 539 amino acids (62.7 kDa), which contained sequence motifs characteristic of RNA-dependent RNA polymerases (RdRps). A database search showed that P1 was closely related to RdRps of members of the genus *Partitivirus* in the family *Partitiviridae*. RNA S2 had the potential to encode a protein, P2, of 430 amino acids (46.3 kDa), which was related to capsid proteins of members of the genus *Partitivirus*. Virus particles isolated from isolate HP62 were shown to be isometric with a diameter of 30 nm, and to contain dsRNAs S1 and S2 and a single capsid protein of 46 kDa. N-terminal sequencing of tryptic peptides derived from the capsid protein proved unequivocally that it is encoded by RNA S2 and corresponds to protein P2. It is concluded that *O. himal-ulmi* isolate HP62 contains a new

member of the genus *Partitivirus*, which is designated *Ophiostoma partitivirus 1*. A phylogenetic tree of RdRps of members of the family *Partitiviridae* showed that there are at least two RdRp lineages of viruses currently classified in the genus *Partitivirus*. One of these lineages contained viruses with fungal hosts and viruses with plant hosts, raising the possibility of horizontal transmission of partitiviruses between plants and fungi. The partitivirus RdRp and capsid proteins appear to have evolved in parallel with the capsid proteins evolving much faster than the RdRps.

### Introduction

Viruses have been developed as biological control agents for *Cryphonectria parasitica*, the fungus that causes chestnut blight [1, 2], and have potential for the control of other plant pathogenic fungi. As part of our programme aimed at developing viruses for control of fungi pathogenic to forest trees, we have discovered and characterized viruses in the Dutch elm disease fungi, *Ophiostoma ulmi* and *O. novo-ulmi*. These viruses consist of a single molecule of naked double-stranded RNA, located in the mitochondria, and have been placed in the genus *Mitovirus* in the family *Narnaviridae* [3–5].

We now report the discovery of a new virus in the third Dutch elm disease fungus, *O. himal-ulmi* [6]. We have isolated and characterized the virus particles and their dsRNA genome. The sequence and genome organization of this virus show that it can be classified in the genus *Partitivirus* of the family *Partitiviridae* and

---

L. J. Crawford · T. A. M. Osman · R. H. A. Coutts (✉) ·  
K. W. Buck  
Division of Biology, Faculty of Life Sciences,  
Imperial College London, Sir Alexander Fleming Building,  
London SW7 2AZ, UK  
e-mail: r.coutts@imperial.ac.uk

F. P. Booy  
Division of Molecular Biosciences, Faculty of Life Sciences,  
Imperial College London, Wolfson Building,  
London SW7 2AZ, UK

C. M. Brasier  
Forest Research Agency, Alice Holt Lodge, Farnham,  
Surrey GU10 4LH, UK

this is the first demonstration of a partitivirus in the genus *Ophiostoma*.

## Materials and methods

### Fungal isolate

*O. himal-ulmi* isolate HP62 has been described previously [6] and was maintained on malt extract agar (MEA, Oxoid) as described [7].

### DsRNA isolation

Total nucleic acids were isolated as described [8] from fungal mycelium after 14 days growth on cellophane membranes overlaying MEA at 22°C. Single-stranded (ss) RNA and dsRNA were separated by precipitating ssRNA with 2 M LiCl and dsRNA with 5 M LiCl as described [8]. DsRNAs were separated by polyacrylamide gel electrophoresis [9] and extracted from the gel using an RNaid kit (Bio 101, Vista, CA, USA).

### cDNA synthesis, cloning and sequence analysis

cDNA libraries were constructed using gel-purified dsRNA segments S1 and S2 from *O. himal-ulmi* isolate HP62. cDNA synthesis, cloning and screening of cDNA clones were carried out as described [8]. Determination of the ends of each dsRNA was done using a GIBCO BRL (Gaithersburg, MD, USA) 5' RACE system [9]. Sequences of cDNA clones were obtained by the chain termination method [10] using dye-terminator cycle sequencing with AmpliTaq DNA polymerase FS (ABI PRISM 377). Sequence analysis was performed using the GCG version 8.1 programs [11]. Multiple sequence alignments, construction of phenograms, and bootstrapping analysis were accomplished with the CLUSTAL X programs [12]. The phylogenetic tree was displayed using the program TREEVIEW [13]. Incubations with S1 nuclease and DNase 1 were as described [3].

### Isolation and purification of virus particles

Actively growing mycelium (60 g) was disrupted by grinding using a mortar and pestle with carborundum (6 g) and sufficient PBK buffer (0.03 M sodium phosphate, 0.3 M potassium chloride, pH 7.6) to form a smooth paste. The homogenate was diluted with PBK buffer to give a volume of 1.8 l, gently shaken at 4°C for 15 min and then centrifuged at 23000g for 45 min.

To the supernatant were added 10 g polyethylene glycol-6000 and 1 g NaCl per 100 ml, and the mixture was stirred at 4°C for 2 h. The precipitate was collected by centrifugation at 15000g for 20 min and suspended in 100 ml PBK buffer. After centrifugation at 23000g for 20 min to remove unsuspended debris, the supernatant was centrifuged at 78000g to pellet the virus. The pelleted virus was resuspended in 1 ml PBK buffer and purified by sequential sedimentation through two 10–50% (w/v) sucrose density gradients. The virus band, identified by a blue-grey opalescence, was collected and dialyzed against 10 mM Tris-HCl, pH 7.6 buffer.

### Electron microscopy

A 5- $\mu$ l drop of virus suspension was placed on a carbon-collodion-coated grid and stained with 1% aqueous uranyl acetate. Grids were examined in a Philips CM 200 electron microscope and images were recorded at 50,000 $\times$  magnification.

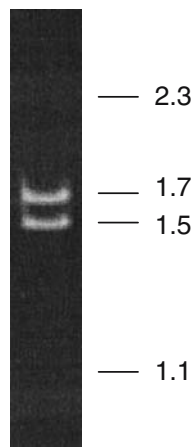
### Protein analysis

Virus particles were disrupted by mixing with one tenth volume of 10% SDS and heating to 100°C for 5 min. 0.5 volume of buffer containing 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and 20% (v/v) glycerol was added and the sample heated to 100°C for 5 min. The protein was analysed by electrophoresis through 10% polyacrylamide gels [14]. Protein digestion with trypsin, separation of tryptic peptides by high pressure liquid chromatography (HPLC) and N-terminal sequencing by Edman degradation were carried out by Dr. John Leszyk at the University of Massachusetts Medical School Worcester Foundation Campus Core laboratory for Protein Microsequencing and Mass Spectrometry.

## Results

### Isolation and characterization of dsRNAs from *O. himal-ulmi*

Total nucleic acids were extracted from *O. himal-ulmi* isolate HP62, ssRNA was precipitated with 2 M LiCl and dsRNA was precipitated with 5M LiCl. Analysis by polyacrylamide gel electrophoresis (PAGE) revealed two RNA segments, S1 and S2, of about 1.7 and 1.5 kbp, respectively (Fig. 1). Both RNA segments were resistant to S1 nuclease and DNase I (not shown), confirming that they are dsRNA.



**Fig. 1** Polyacrylamide gel electrophoresis of dsRNA from *O. himal-ulmi* isolate HP62. The gel was stained with ethidium bromide. The sizes of dsRNA markers (kbp) from *O. novo-ulmi* isolate Ld [4] are shown on the side of the gel

The complete nucleotide sequence of each dsRNA segment was determined from cDNA clones obtained using random primers, sequence-specific primers and 5' RACE. Segment S1 comprised 1744 bp and segment S2 comprised 1567 bp. The EMBL database accession numbers for these sequences are S1 (AM087202) and S2 (AM087203). Segment S1 had the potential to encode a protein, P1, of 539 amino acids (calculated mass 62.7 kDa) on one strand, designated the plus strand. Segment S2 had the potential to encode a protein, P2, of 430 amino acids (calculated mass 46.3 kDa) on one strand, designated the plus strand. There were no significant open reading frames on the complementary (minus) strands of either RNA segment. The 5' untranslated regions of the plus strands of segments S1 and S2 were 69 and 108 nucleotides, respectively, whereas the 3' untranslated regions of the plus strands of segments S1 and S2 were 166 and 55 nucleotides, respectively. The 5'-terminal sequences of the two RNAs were almost identical, ACCGAAA for S1 and AUCGAAA for S2. Similarly there was a high degree of sequence similarity between the 3'-terminal sequences, with both segments containing the sequence UAUCAGG. This is consistent with evidence that for multicomponent RNA viruses, the 5'- and 3'-terminal sequences are important as recognition sites for the viral RNA RdRp [15].

A search of the UniProt database with the BLASTP program using the P1 amino acid sequence revealed highly significant similarities to RNA-dependent RNA polymerases (RdRps) of viruses of the family *Partitiviridae*. The most similar were the RdRps of *Discula destructiva viruses 1 and 2* (DdV1 and DdV2) (71% and 71% identity, respectively), *Penicillium stoloniferum*

*virus S* (PsV-S) (67% identity), *Gremmeniella abietina virus MS1* (GaV-MS1) (66% identity), and *Fusarium solanae solani virus* (FsV). Analysis of the amino acid sequence of the P1 protein revealed motifs typical of RdRps, which could be aligned with similar motifs of a range of partitiviruses (Fig. 2). Hence the protein P1, encoded by *O. himal-ulmi* dsRNA S1, is a putative RdRp of a partitivirus, designated *Ophiostoma partitivirus 1* (OPV1). A phylogenetic tree of the RdRps of these partitiviruses (Fig. 3) shows that the OPV1 RdRp clusters with the RdRps of DdV1, DdV2, PsV-S, GaV-MS1 and FsV with 100% bootstrap support.

A search of the UniProt database with the BLASTP program using the P2 amino acid sequence revealed highly significant similarities to the capsid proteins of the partitiviruses DdV1 (52% identity), DdV2 (51% identity), PsV-S (51% identity), GaV-MS2 (51% identity), GaVMS1 (50% identity), and FsV (39% identity). An amino acid alignment of these six capsid proteins (Fig. 4) showed that they have identical amino acids at 83 positions. Hence the protein, P2, encoded by *O. himal-ulmi* dsRNA segment S2, is the putative capsid protein of the partitivirus OPV1.

#### Isolation and characterization of OPV1 virus particles

OPV1 virus particles were isolated from homogenates of mycelium of *O. himal-ulmi* isolate HP24 by PEG precipitation, and differential and sucrose density gradient centrifugation. Electron microscopy revealed isometric particles of average diameter 29.7 nm (SD 1.8 nm;  $n = 30$ ) (Fig. 5). Examination of dsRNA extracted from the purified virions by PAGE revealed two bands with the same mobility as those obtained by extraction of total RNA from *O. himal-ulmi* (not shown). Sequence analysis of these two RNAs showed that they are identical to RNAs S1 and S2. Examination of the OPV1 capsid protein by SDS-PAGE revealed a single band with an estimated mass of 46 kDa (not shown), the same mass as calculated for the protein encoded by *O. himal-ulmi* dsRNA segment S2. The 46-kDa band was extracted from a gel, blotted onto a membrane and digested with trypsin. The tryptic peptides were separated by HPLC and their masses determined by mass spectrometry. The N-terminal sequences of two of the peptides were determined by Edman degradation. Peptide 1 (mass 1372.48 Da) had the sequence SAVSSAPGAP-ASNQK, which corresponds to amino acids 29–43 of the protein P2 encoded by OPV1 dsRNA segment S2. Peptide 2 (mass 1435.63 Da) had the sequence FPVVFATGAGEPSR, which corresponds to amino

RdRp	1	2	3	4	5	6
OPV1	<b>PKTRLVW</b> 54	<b>DFSSFDTKVP</b> 61	<b>GVPSGSWWTQ</b> MIDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGVK</b> 7	<b>RPTLEW</b>
DdV1	<b>PKTRLVW</b> 54	<b>DFSAFDSKVP</b> 61	<b>GVPSGSWWTQ</b> IIDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGTK</b> 7	<b>RSTDEW</b>
DdV2	<b>PKTRLIW</b> 54	<b>DFSAFDSKVP</b> 61	<b>GVPSGSWWTQ</b> IIDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGTK</b> 7	<b>RSTDEW</b>
PsV-S	<b>PKTRLVW</b> 54	<b>DFSGFDTKVP</b> 61	<b>GVPSGSWFTQ</b> MIDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGTT</b> 7	<b>RETNEW</b>
GaVMS1	<b>PKTRLVW</b> 54	<b>DFSSFDTKVP</b> 61	<b>GVPSGSWWTQ</b> MVDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGTT</b> 7	<b>RDITNEW</b>
FsV	<b>PKTRLAW</b> 54	<b>DFSSFDTKVP</b> 61	<b>GVPSGSWWTQ</b> LVDSVVN 19	<b>VLGDDS</b> 38	<b>KLLGVR</b> 7	<b>RETEW</b>
PsV-F	<b>LKTRAVW</b> 57	<b>DWSGFDTSVS</b> 56	<b>GIPSGSFFTQ</b> IIGSIVN 19	<b>VLGDDS</b> 41	<b>KFLGYQ</b> 7	<b>RSTTEW</b>
HmV	<b>TKIRVVF</b> 61	<b>DWSSEFDLRS</b> L 60	<b>GIPSGLEF</b> TTQFLDSFYN 21	<b>VQGD</b> DS 46	<b>NALGYD</b> 7	<b>RDWRKL</b>
HaV	<b>NKLR</b> TIW 59	<b>DWSRF</b> DKRAY 65	<b>GIPSG</b> LLFITQMLDSWYN 21	<b>WLG</b> DDS 46	<b>EVL</b> SYR 7	<b>RDLLKM</b>
RnV1	<b>LKQ</b> RPVY 59	<b>DWSSE</b> FQRPV 72	<b>GVPSG</b> LLNTQYLDSFCN 24	<b>IMG</b> DDN 44	<b>ETL</b> SYQ 7	<b>RPLGKL</b>
AhV	<b>LKVR</b> PVY 54	<b>DWSRF</b> DHLAP 91	<b>GVPSG</b> ILMTQFIDSFVN 24	<b>IMG</b> DDN 44	<b>EVL</b> GYT 7	<b>RSISK</b> L
FpV	<b>LKQ</b> RPVY 54	<b>DWSGF</b> DQRLP 72	<b>GVPSG</b> MLNTQFLDSFGN 24	<b>IMG</b> DDN 44	<b>ETL</b> SYQ 7	<b>RPIGKL</b>
RsV	<b>LKVR</b> PVY 54	<b>DWSGYD</b> QRLP 71	<b>GVPSG</b> MLLTQFLDSFGN 24	<b>IMG</b> DDN 44	<b>ETL</b> SYR 7	<b>RDVEKL</b>
OMV	<b>PKVR</b> LVF 55	<b>DWSGF</b> DRYAR 74	<b>GIYSG</b> YMQTQILDLYN 21	<b>VQGD</b> DS 39	<b>DSL</b> EHA 7	<b>RNGE</b> IPY
PoV	<b>LKQ</b> RPVY 55	<b>DWSGF</b> DQRLP 72	<b>GVPSG</b> LLNTQYLDSFSN 24	<b>VMG</b> DDN 43	<b>ETL</b> SYQ 7	<b>RPLAK</b> L
CrV	<b>LKVR</b> PNV 55	<b>DWSKF</b> DQTV 93	<b>GVPSG</b> ILCTQLIDSFVN 24	<b>LMG</b> DDN 44	<b>ETL</b> GYT 7	<b>RSLSK</b> L
CCRSAV	<b>NKMRT</b> IW 60	<b>DWKRF</b> DKKAY 71	<b>GIPSG</b> LEFITQLIDSWYN 21	<b>VGG</b> DS 46	<b>EVL</b> SYR 7	<b>RDEL</b> AM
BCV	<b>TKVR</b> GVW 55	<b>DWSSE</b> FSSVT 50	<b>GIPSG</b> SYTTSIVGSVVN 20	<b>TQG</b> DS 38	<b>TFL</b> GRT 7	<b>RSLDK</b> C
PpV	<b>TKIR</b> NVW 54	<b>DWKQF</b> DATVS 50	<b>GIPSG</b> SYFTSIIGSIIN 20	<b>TQG</b> DS 38	<b>HFL</b> GRT 7	<b>REIK</b> R
FCCV	<b>RKIR</b> NVW 55	<b>DWSGF</b> DASVQ 50	<b>GIPSG</b> SCFTNIIGSIVN 19	<b>THG</b> DS 40	<b>SFL</b> SRK 7	<b>RDEL</b> LC
RYEV	<b>PKIR</b> TVF 60	<b>DWSEF</b> DMRVY 66	<b>GMPSG</b> IFCTQFWDSEFYN 22	<b>VLGD</b> DV 46	<b>QVL</b> SYI 7	<b>RDSN</b> QL
WCCV	<b>NKMRT</b> IW 60	<b>DWSRF</b> DKRAY 60	<b>GIPSG</b> LEFITQLLDSEWYN 21	<b>VQGD</b> DS 46	<b>EVL</b> SYR 7	<b>RDEIT</b> M
	* *	* *	* ** *	***	*	*

**Fig. 2** Conserved amino acid sequence motifs in RdRps of partitiviruses. Motifs 2, 3, 4, and 5 were previously designated IV–VII by Koonin [16]. Motifs 1–5 were previously designated 3–7 by Bruenn [17]. Amino acids identical to those in the *O. himal-ulmi* P1 protein are shown in bold. Amino acids identical in all the sequences are shown by a star (\*) below the sequences. The numbers of amino acids between each motif for each RdRp are indicated. Abbreviations, with Uniprot database accession numbers in parentheses, are: OPV1, *Ophiostoma partitivirus 1*; DdV1, *Discula destructiva virus 1* (Q99D1); DdV2, *Discula destructiva virus 2* (Q91H18); PsV-S, *Penicillium stoloniferum virus S* (Q6YDQ7); GaV-MS1, *Gremmeniella abietina virus MS1* (Q6GYB9); FsV, *Fusarium solanae virus*

(Q83328); PsV-F, *Penicillium stoloniferum virus F* (Q4G3H2); HmV, *Helicobasidium mompa virus* (Q8B0U3); HaV, *Heterobasidium annosum virus* (Q91T12); RnV1, *Rosellinia necatrix virus* (Q50LH7); AhV, *Atkinsonella hypoxylon virus* (Q85055); FpV, *Fusarium poae* (Q9YX75); RsV, *Rhizoctonia solania solani virus* (Q9Q9X2); OMV, *Oyster mushroom virus* (Q7TDZ9); PoV, *Pleurotus ostreatus virus* (Q5GHF1); CrV, *Ceratocystis resinifera virus* (Q5DM90); CCRSAV, *Cherry chlorotic rusty spot associated virus* (Q65A70); BCV, *Beet cryptic virus* (Q86632); PpV, *Pyrus pyrifolia virus* (Q92WP7); FCCV, *Fragaria chiloensis cryptic virus* (Q4FCM7); RYEV, *Radish yellow edge virus* (Q52UL1); WCCV, *White clover cryptic virus* (Q64FP0)

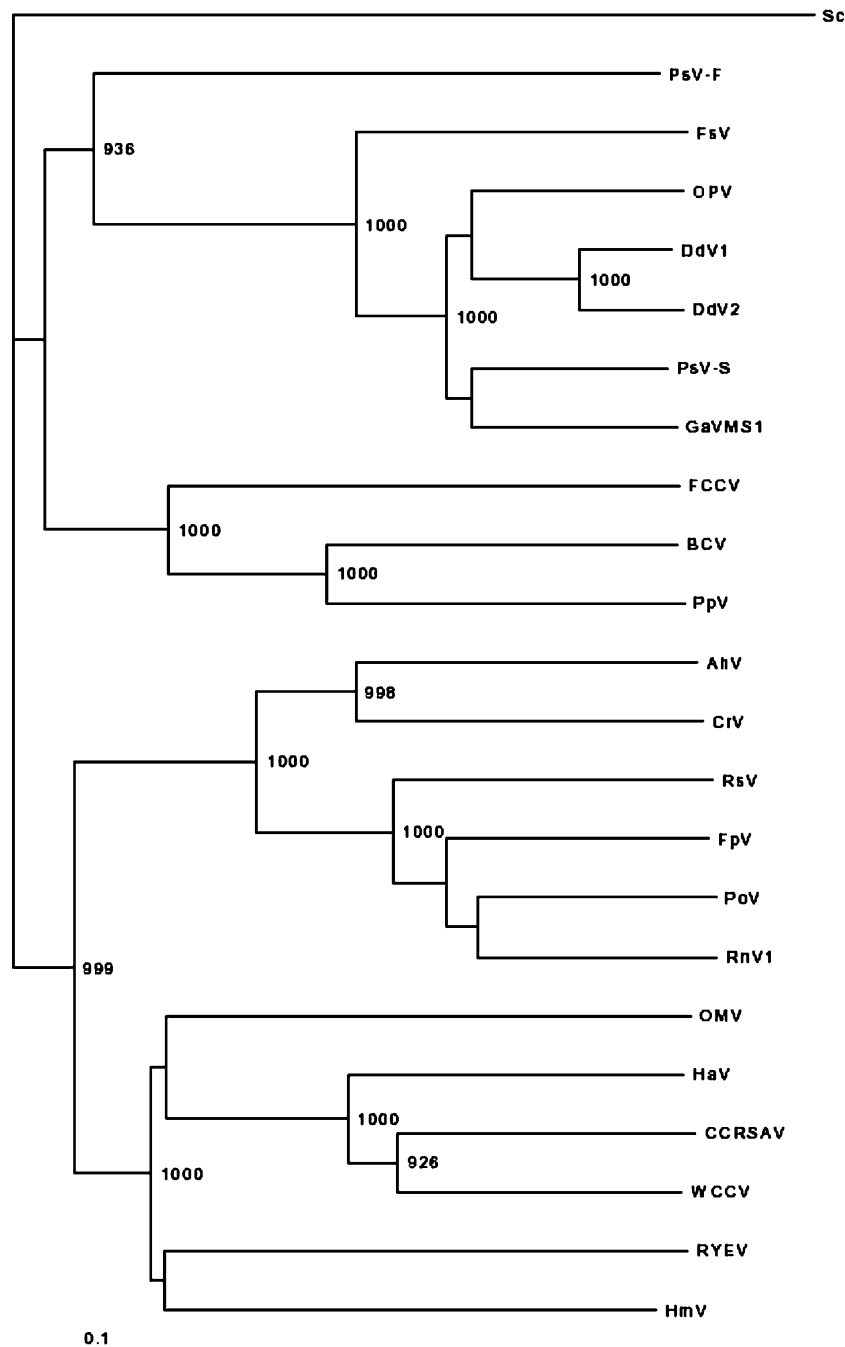
acids 63–76 of the protein P2. This proves conclusively that dsRNA segment S2 encodes the OPV1 capsid protein.

## Discussion

The family *Partitiviridae* consists of viruses with isometric virions, 30–35 nm in diameter, a single major capsid protein of 42–73 kDa and a genome of two dsRNA segments each of 1.4–2.2 kbp [18]. Three genera in the family *Partitiviridae* have been described; the genus *Partitivirus* contains viruses of fungi, whereas the genera *Alphacryptovirus* and *Betacryptovirus* contain viruses of plants. The data reported in this paper clearly show that OPV1 is most closely related to fungal viruses in the genus *Partitivirus*. In the RdRp phylogenetic tree (Fig. 3), OPV1 and established fungal virus members of the genus *Partitivirus*, DdV1, DdV2, PsV-S, GaV-MS1 and FsV, form a cluster with 100% bootstrap support. The

capsid protein sequences of these viruses were also closely related (Fig. 4), although less so than the RdRps, indicating a higher rate of evolution of the capsid proteins. There is a second well-supported fungal virus RdRp cluster, consisting of AhV, CrV, RsV, FpV, PoV and RnV1 (Fig. 3). The capsid proteins of viruses in the first RdRp cluster were not significantly related to those in the second RdRp cluster. The RdRp and capsid protein sequences of these two clusters therefore appear to have evolved in parallel and viruses in the two clusters may be regarded as two subgroups of the genus *Partitivirus*.

There are also two other well-supported RdRp clusters, one consisting of BCV (genus *Alphacryptovirus*) and two other plant viruses (FCCV and PpV) and the other consisting of a mixture of plant viruses (WCCV and RYEV, genus *Alphacryptovirus*) and fungal viruses (OMV, HaV and HmV, genus *Partitivirus*), together with CCRSAV, which may be a fungal or plant virus [19]. These two clusters also form a larger cluster with good (99.9%) bootstrap support.



**Fig. 3** Neighbour-joining phylogenetic tree constructed using the RdRp sequences shown in Fig. 2, including the sequences between the motifs, using the program CLUSTAL X. Abbreviations are as in Fig. 2. Bootstrap numbers out of 1,000 replicates are given on the nodes. Only numbers greater than 900 are

shown. The tree was rooted with the RdRp of *Saccharomyces cerevisiae virus L-A* (ScVLA), a member of the genus *Totivirus* in the family *Totiviridae* [Uniprot database accession no. Q87023], which was included as an outgroup

This raises the possibility of horizontal transfer of members of the family *Partitiviridae* between fungi and plants [20, 21]. This is quite feasible, since some of the viruses in these clusters have fungal hosts,

which are pathogens of plants. The taxonomy of the *Partitiviridae* family may need to be revisited as molecular information of a wider range of viruses becomes available.



11. J. Devereux, P. Haerberli, O. Smithies, *Nucleic Acids Res.* **12**, 539–549 (1984)
12. J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, *Nucleic Acids Res.* **24**, 4876–4882 (1997)
13. R.D.M. Page, *Comput. Applic. Biosci.* **12**, 357–358 (1996)
14. U.K. Laemmli *Nature* **227**, 680–685 (1970)
15. K.W. Buck, *Adv. Virus Res.* **47**, 159–251 (1996)
16. E.V. Koonin, *J. Gen. Virol.* **72**, 2197–3206 (1991)
17. J.A. Bruenn, *Nucleic Acids Res.* **21**, 5667–5669 (1993)
18. S.A. Ghabrial, K.W. Buck, B.I. Hillman, R.G. Milne, in *Virus Taxonomy, VIIIth Report of the ICTV*, eds by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Elsevier/Academic Press, London, 2004) pp. 581–590
19. R.H.A. Coutts, L. Covelli, F. Di Serio, A. Citir, S. Açıkgöz, C. Hernández, A. Ragozzino, R. Flores, *J. Gen. Virol.* **85**, 3399–3408 (2004)
20. K.W. Buck, in *Molecular variability of fungal pathogens*, ed. by P.D. Bridge, Y. Couteaudier, J.M. Clarkson (CAB International, Wallingford, 1998) pp. 53–72
21. S.A. Ghabrial, *Virus Genes* **16**, 119–131 (1998)